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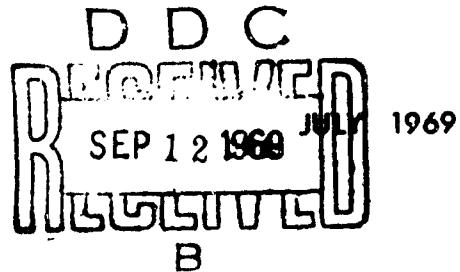
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TECHNICAL MANUSCRIPT 546

INTERACTION OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS WITH NEUTRALIZING ANTIBODY:

III. THE SERUM NEUTRALIZATION ANTI-IgG TEST

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III. THE SERUM NEUTRALIZATION ANTI-IgG TEST

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In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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ABSTRACT

A precise, reproducible, and sensitive serum neutralization test was developed to estimate Venezuelan equine encephalomyelitis (VEE) serum-neutralizing antibodies within 24 hours. The test is dependent on both the interactions of virus with antiviral serum and the resultant complex with anti-gamma globulin (IgG) antibodies. The 50% serum-neutralizing end point was calculated from the reduction of number of fluorescent cells in McCoy cell monolayers resulting from the neutralization of infective viral particles by antibodies. In comparative estimates of neutralizing antibodies of human and monkey sera, the sensitivity of the serum neutralization anti-IgG test was several hundred times greater than that of the conventional mouse serum neutralization test.

I. INTRODUCTION*

Recently, a quantitative assay of Venezuelan equine encephalomyelitis (VEE) virus was developed that is based on the enumeration of cells containing fluorescent viral antigen.¹ This assay system proved highly suitable for characterizing the in vitro interaction between virus and neutralizing antibody.² In the course of these studies, we noted that neutralization reactions were initially first order and then followed curvilinear kinetics even in the presence of excess antiviral serum. This anomalous behavior, attributed to a small fraction of a viral population that resists neutralization, was caused by the formation of an infectious virus - antibody complex (sensitized virus). The complex could be neutralized by anti-IgG serum, an antibody not directed toward and inactive against the virus.³ An attempt was made, therefore, to increase the sensitivity of the conventional serum neutralization test by incorporating anti-IgG serum with the reactants, virus and antiviral serum.

This report describes the development and standardization of a serum neutralization anti-IgG test for VEE that is dependent on both the interactions of virus with antiviral serum and the resultant complex with anti-IgG antibodies.

II. MATERIALS AND METHODS

A. VIRUS AND ANTIVIRAL SERUM

The source and preparation of the Trinidad strain of VEE virus were described previously.¹

VEE antisera were obtained from rhesus monkeys that had been exposed 1 month earlier to an aerosol of approximately 1,000 cell-infecting units (CIU) of virus. Human antiviral sera were obtained from immunized personnel and from a convalescent patient. Sera were inactivated at 56 C for 30 minutes.

B. ANTI-GAMMA GLOBULIN (IgG) SERUM

Goat anti-monkey IgG serum was obtained from Microbiological Associates, Bethesda, Maryland. Normal goat serum was purchased from Pentex Laboratories, Kankakee, Illinois.

* This report should not be used as a literature citation in material to be published in the open literature. Readers interested in referencing the information contained herein should contact the author to ascertain when and where it may appear in citable form.

C. CELL LINE

The McCoy cell line was used for assay of unneutralized virus particles. Nutrient medium for the cells consisted of medium 199 containing 0.5% (w/v) lactalbumin hydrolysate, 10% fetal calf serum, 50 µg of streptomycin per ml, and 75 µg of kanamycin per ml. Cells were maintained in medium 199 plus 5% fetal calf serum. They were cultivated on circular cover slips (15-mm diameter) inserted in flat-bottomed glass vials (19 by 65 mm). One milliliter of cell suspension containing 10^5 to 3×10^5 cells was introduced onto cover slips, which were then incubated at 35°C for 24 hours, or until a complete cell monolayer was formed. Cell monolayers were washed in phosphate-buffered saline (PBS), pH 7.1, free of calcium and magnesium ions, before the addition of inoculum.

D. SERUM NEUTRALIZATION ANTI-IgG TEST

The general procedure consisted of mixing appropriate dilutions of antiviral or normal serum with equal volumes of a constant quantity of virus (7.4×10^4 CIU/ml). Virus and serum dilutions were routinely prepared in PBS. Test mixtures were incubated at 35°C for 2 hours. To each test mixture, a 1/2 dilution of goat anti-monkey IgG serum was added in a volume to give a final dilution of 1/6. After incubation at 35°C for 10 minutes, 0.2 ml of each mixture was introduced onto one of three cover slip cultures of McCoy cells for assay of unneutralized virus.

The technique based on immunofluorescent cell counting was employed for assay of virus and is described in detail elsewhere.¹ Briefly, the procedure consisted of centrifuging inoculum onto cell monolayers at 19,642 to 29,432 x g for 15 minutes. Residual fluid was then removed and 1 ml of maintenance medium was added. After incubation of cover slip monolayers at 35°C for 1 hour to ensure virus penetration into host cells, cell monolayers were held at 35°C for 20 hours in the presence of 1 ml of overlay consisting of a 1/20 dilution of antiviral serum in medium 199. The overlay precluded the occurrence of a second cycle of infection. If cell monolayers were fixed at 12 hours, the antiviral serum overlay was omitted. Infected cell cultures were fixed with cold (-0°C) acetone and either prepared immediately for immunofluorescent staining and infected-cell counting or stored at -60°C.

E. IMMUNOFLUORESCENCE PROCEDURES

Rhesus monkey VEE antiserum was conjugated with fluorescein isothiocyanate;⁴ unbound dye was removed from conjugated globulin by passing the globulin through a column of Sephadex G-25. To reduce nonspecific fluorescence, 5 ml of conjugated globulin were diluted with an equal volume of PBS and adsorbed twice with 200 mg of acetone-dried mouse liver powder.⁵ The direct method was used to demonstrate immunofluorescence of viral antigens in infected cells. Fixed cell cultures were washed twice with PBS and stained with conjugated antiserum for 30 minutes at room temperature. Cover slip cell monolayers were then rinsed in two changes of PBS and mounted in a semipermanent medium.⁶

An American Optical Co. microscope equipped with a Fluoroluma illuminator (model 645), Corning No. 5840 and Schott BG-12 exciter filters, and an E.K. No. 2A barrier filter was used to examine stained cover slip cell monolayers. With this optical system at a magnification of 430X, 1,064 microscopic fields could be seen in the area of a 15-mm cover slip. For each cover slip cell monolayer, 50 microscopic fields were examined for fluorescent cells. To calculate the number of CIU of virus per milliliter, the average number of fluorescent cells per field was multiplied by the number of fields per cover slip, the reciprocal of the dilution of virus inoculum, and a volume factor (for conversion to milliliters).

To determine the 50% serum-neutralizing end point, the per cent reduction of fluorescent cell counts for each antiserum dilution was computed from control counts. Reduction percentages were then plotted against the logarithm of the corresponding final dilutions of antiserum on probability paper. A linear relationship was obtained over a critical range. By interpolation, the dilution of antiserum that neutralized 50% of virus was determined.

F. MOUSE SERUM NEUTRALIZATION TEST

In trials that compared this method and the serum neutralization anti-IgG test, a similar protocol and similar conditions for reacting virus and antiviral serum were used, e.g., virus concentration, incubation time, volume of inoculum. However, instead of goat anti-monkey IgG serum, normal goat serum was added to each virus-antiviral serum mixture. For each test mixture, eight Swiss mice, weighing 10 to 14 g, were inoculated intraperitoneally with 0.2 ml. Animals were observed daily for 10 days and the number of survivors was recorded. The 50% serum-neutralizing end point was calculated by the Reed and Muench formula.⁷

III. RESULTS

A. PARAMETERS OF NEUTRALIZATION

Parallel tests were performed to determine the 50% neutralizing titer of VEE monkey antiviral serum in the presence of goat antiserum to monkey IgG or normal goat serum. Experimental details are described in Section II. Results (Fig. 1) show that the 50% serum-neutralizing end points in the presence of anti-IgG or normal goat sera were 1/105,000 and 1/40,000, respectively. The serum-neutralizing titer was more than twofold higher when anti-IgG serum was incorporated into the virus-antiviral serum mixtures.

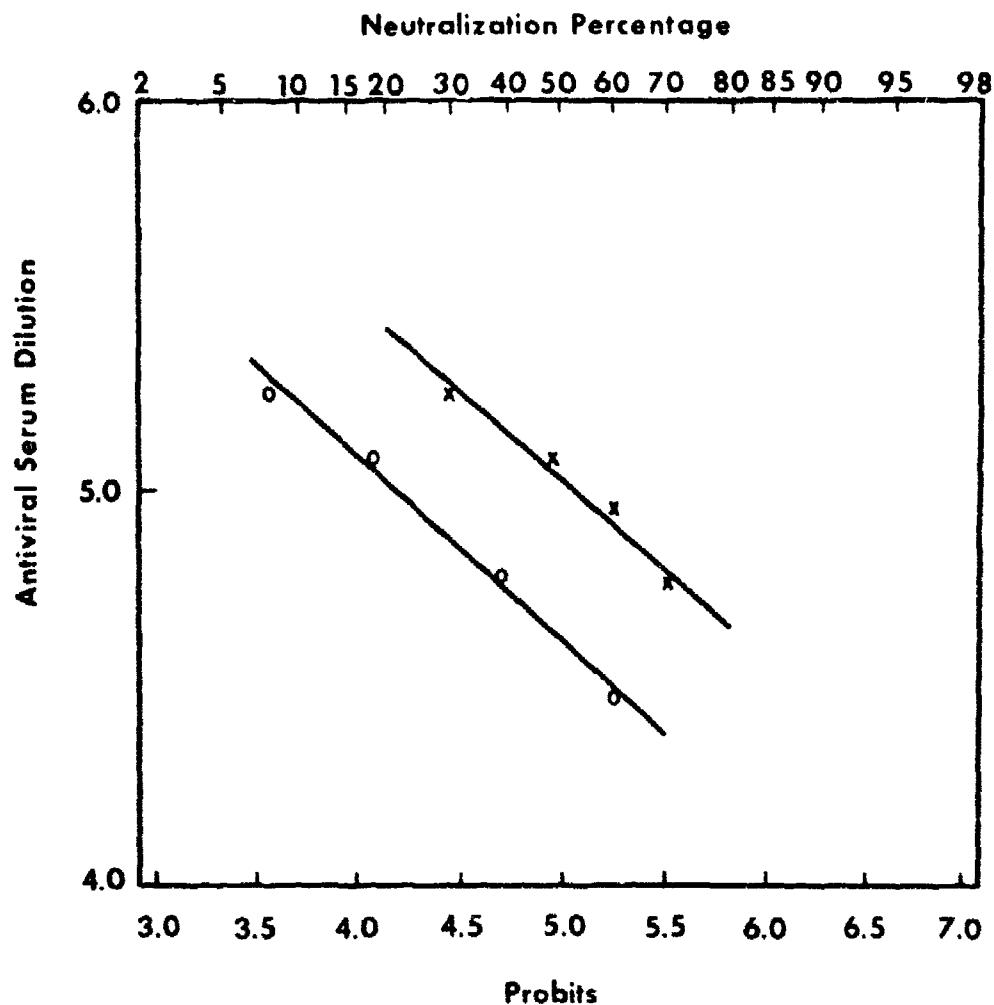


FIGURE 1. Comparative Determination Between the Serum Neutralization Anti-IgG Test (X) and a Conventional Serum Neutralization Test (O). Fifty per cent serum-neutralizing end points were interpolated from the per cent reduction of VEE virus - infected cells by antiviral serum dilut.

A series of experiments, using the serum neutralization anti-IgG test, was carried out to assess the influence of various conditions and reagent concentrations on the serum-neutralizing titer. Neutralization tests were carried out under different conditions of incubation for virus-antiviral serum mixtures and for the reaction of mixtures with anti-IgG serum. Results in Table 1 indicate that 50% serum neutralizing titers were comparable whether virus-antiviral serum mixtures were incubated at 35 C for 2 hours or for longer time periods. The additional factor of incubation with anti-IgG serum for varied time intervals and at different temperatures did not affect serum-neutralizing end points. Evidence that temperatures above 35 C may markedly affect serum-neutralizing titers was noted when virus-antiviral serum mixtures were incubated at either 35 C or 50 C. Serum-neutralizing end points were approximately twice as high at 50 C than at 35 C for the same incubation time of $\frac{1}{2}$ hour. At 50 C neutralizing titers were comparable to those obtained at incubation conditions of 35 C for 2 hours. The reactants held at 50 C, however, appeared to attain equilibrium within one-fourth the time. In subsequent tests, virus-antiviral serum mixtures were routinely incubated at 35 C for 2 hours and then at 35 C for 10 minutes with anti-IgG serum.

TABLE 1. EFFECT OF INCUBATION CONDITIONS FOR VEE VIRUS, MONKEY ANTIVIRAL, AND ANTI-IgG SERUM MIXTURES ON THE SERUM-NEUTRALIZING END POINT

Virus and Antiserum	Incubation	Anti-IgG ^a /	50% Serum Neutralizing End Point ^b /
35 C, $\frac{1}{2}$ hour		35 C, 10 minutes	56,000
35 C, 2 hours		35 C, 10 minutes	115,000
35 C, 2 hours		35 C, 1 hour	110,000
35 C, 4 hours		35 C, 1 hour	90,000
35 C, 2 hours		25 C, 10 minutes	110,000
4 C, 26 hours		35 C, 10 minutes	110,000
4 C, 26 hours; 35 C, 2 hours		35 C, 10 minutes	105,000
50 C, $\frac{1}{2}$ hour		35 C, 10 minutes	120,000

a. Goat antiserum to monkey IgG.

b. Reciprocal of antiviral serum dilution.

Because previous studies showed that the union of sensitized virus (infectious virus - antibody complex) with anti-IgG antibodies occurs within ½ minute,³ virus, antiserum, and anti-IgG serum were initially mixed together and incubated at 35°C for 2 hours to determine whether virus neutralization could occur effectively. Thus, it could be possible to eliminate the secondary incubation period now used for anti-IgG interaction with sensitized virus. Normal goat serum incorporated into mixtures was also tested under similar circumstances. The usual neutralization test was also carried out with virus-antiviral serum mixtures incubated at 35°C for 2 hours. When either anti-IgG or normal goat serum was added, the mixtures were incubated additionally at 35°C for 10 minutes. The 50% serum-neutralizing end points were determined for each test.

When the reactants (virus, antiviral serum, and anti-IgG serum) were initially mixed and incubated, the serum-neutralizing titer was approximately 50-fold lower than that of mixtures containing normal goat serum (Table 2). The serum-neutralizing titer was the same when normal goat serum was added to mixtures initially or after the primary incubation period. Anti-IgG serum added to virus-antiviral serum mixtures after the primary incubation period resulted in a 150-fold higher serum-neutralizing titer than that obtained by mixing these reactants together before incubation. These data emphasize the importance of first incubating virus-antiviral mixtures for a suitable time period before adding anti-IgG serum.

TABLE 2. EFFECT OF SEQUENCE OF MIXING REACTANTS AND THEIR INCUBATION ON VEE SERUM-NEUTRALIZING END POINT

<u>Initial Mixture^{a/}</u>	<u>Serumb/ Additive</u>	<u>50% Serum- Neutralizing End Point^{c/}</u>
Virus, antiviral + anti-IgG sera	None	700
Virus, antiviral + normal goat sera	None	40,000
Virus + antiviral serum	anti-IgG	105,000
Virus + antiviral serum	normal goat	40,000

- a. Monkey antiviral serum and goat antiserum to monkey anti-IgG were used.
Mixtures were incubated at 35°C for 2 hours.
- b. Incubated additionally at 35°C for 10 minutes.
- c. Reciprocal of antiviral serum dilution.

Different virus concentrations were reacted with antiviral and anti-IgG sera mixtures to determine the effect on the serum-neutralizing end point. Neutralization tests were performed in the manner described previously. Tests differed only in the quantity of virus that was reacted with appropriate dilutions of antiviral serum. Results ($Ta^{41}~3$) show that differences in virus concentration, as much as 1.0 log unit, did not significantly change the 50% serum-neutralizing titer. This finding reaffirms the operation of the "percentage law" described by Andrewes and Elford.^a

TABLE 3. EFFECT OF VEE VIRUS CONCENTRATION ON THE SERUM-NEUTRALIZING END POINT

Virus Concentration ^{a/}	Reduction of Control Infected Cell Count, %, at Indicated Antiviral Serum Dilution			50% Serum-Neutralizing End Point ^{b/}
	1/60,000	1/90,000	1/120,000	
3.3×10^4	75	54	37	98,000
1.6×10^4	70	51	43	100,000
6.5×10^3	84	60	34	100,000
3.3×10^3	70	60	43	108,000

a. Cell-infecting units of virus per 0.2 ml of test mixture (antiviral and anti-IgG sera).

b. Reciprocal of antiviral serum dilution.

To determine the influence of different dilutions of anti-IgG on the serum-neutralizing titer, a constant virus quantity was mixed with appropriate antiviral serum dilutions and incubated at 35°C for 2 hours. Sets of representative portions of each virus-antisera mixture were then prepared and, to each set, a different dilution of anti-IgG serum was added. The 50% serum neutralization end point was unchanged when anti-IgG serum dilutions of 1/6 or 1/30 were reacted with virus-antiviral serum mixtures (Table 4). Sufficient anti-IgG antibodies were apparently present in these dilutions to neutralize sensitized virus. With increased dilutions beyond 1/30, the serum-neutralizing titer decreased. To insure that anti-IgG antibodies were available to neutralize varied amounts of sensitized virus present in mixtures, a final dilution of 1/6 of anti-IgG serum was routinely employed in serum neutralization tests.

TABLE 4. EFFECT OF DIFFERENT ANTI-IgG SERUM DILUTIONS ON THE VEE SERUM-NEUTRALIZING END POINT

Anti-IgG Dilutions ^a /	50% Serum- Neutralizing End Point ^b /
1/6	100,000
1/30	100,000
1/60	75,000
1/120	47,000
Normal goat serum	44,000

a. Final dilution of goat anti-monkey IgG serum when added to virus-antiviral serum mixtures that had been previously incubated at 35 C for 2 hours.

b. Reciprocal of antiviral serum dilution.

B. QUANTITATIVE EVALUATIONS

To estimate the precision of the serum neutralization anti-IgG test, ten determinations were made under the same conditions and using the same reagents. The high precision attained is shown in Table 5. The standard deviation ($\pm 5,109$), expressed as a percentage of the mean reciprocal 50% serum-neutralizing titer (107,900), was 4.7. The precision compared favorably with that of the immunofluorescent serum neutralization test for psittacosis.⁹

Serum neutralization tests were performed to determine the reproducibility of the neutralizing end point. Tests were made at different time intervals under the same experimental conditions. The high degree of reproducibility attained (Table 6) indicates that twofold or greater elevations in serum-neutralizing titer may be readily distinguished.

TABLE 5. PRECISION OF THE VEE SERUM NEUTRALIZATION ANTI-IGE TEST

Test	Count ^a / per cent ^b	Final Antiviral Serum Dilution		1/120,000		50% Serum-Neutralizing End Point ^c /	
		1/60,000		1/90,000			
		Reduction, per cent	Count	Reduction, per cent	Count		
1	9	85.0	23	61.7	36	40.0	
2	9	85.0	20	66.7	35	41.7	
3	9	85.0	18	70.0	32	46.7	
4	12	80.0	22	63.4	39	35.0	
5	7	88.4	20	66.7	34	43.4	
6	11	81.7	22	63.4	37	38.4	
7	11	81.7	25	58.2	33	45.0	
8	9	85.0	18	70.0	39	35.0	
9	12	80.0	16	73.4	40	33.4	
10	9	85.0	16	73.4	34	43.4	

a. Infected cells in 50 microscopic fields.

b. Per cent reduction of control infected cell count of 60.

c. Reciprocal of antiviral serum dilution. The arithmetic mean was 107,900 with SD±5,109 and SE of mean ±1,611.

TABLE 6. REPRODUCIBILITY OF VEE SERUM NEUTRALIZATION ANTI-IgG TEST

Test Date	Control Infected Cell Count	Reduction of Control at Indicated Final Antiviral Serum Dilution		50% Serum-Neutralizing End Point ^a /
		1/60,000	1/90,000	
11/20/68	158	70	60	43
11/25/68	306	84	60	34
12/2/68	110	80	65	40
12/9/68	60	82	63	38

a. Reciprocal of antiviral serum dilution.

The sensitivity of the serum neutralization anti-IgG test was compared with that of the conventional mouse serum neutralization test for estimating VEE antibodies of both human and rhesus monkey serum samples. Although neutralization of sensitized virus by anti-IgG serum is generally species-specific, goat anti-monkey IgG serum was employed in the former test because it is equally effective in neutralizing virus sensitized by either human or monkey antiviral serum.³ The experimental protocols used with each test are described in Section II. Significantly higher 50% serum-neutralizing end points were obtained with all sera tested with the use of the serum neutralization anti-IgG test than with the mouse test (Table 7). The average differences obtained between the two tests for estimating serum-neutralizing titers of human and monkey sera were approximately 400- and 300-fold, respectively.

TABLE 7. COMPARATIVE DETERMINATIONS OF VEE NEUTRALIZING ANTIBODIES BY THE SERUM NEUTRALIZATION ANTI-IgG AND MOUSE SERUM NEUTRALIZATION TESTS

Serum Sample	50% Serum-Neutralizing End Point	
	Serum Neutralization Anti-IgG Test	Mouse Serum Neutralization Test
<u>Human Serum^a/</u>		
Convalescent patient	42,000	80
Immunization #28	10,000	<30
Immunization #23	35,000	<30
Immunization #2	6,000	<30
Immunization #4	10,000	62
<u>Rhesus Monkey Serum^a/</u>		
1	100,000	437
2	150,000	447
3	60,000	205
5	45,000	148

a. Reciprocal of antiviral serum dilution. Monkeys exposed to aerosol of VEE virus; serum obtained 30 days later.

IV. DISCUSSION

The serum neutralization anti-IgG test described in this report for estimating VEE neutralizing antibodies is dependent on both the interactions of virus with antiviral serum and the resultant complex with anti-IgG antibodies. The optimal conditions required to effect neutralization of virus in these reactions were defined. Previously, it was shown that although a major portion of the virus population is neutralized by antiviral serum, a small fraction resisted neutralization even in the presence of excess virus antiserum.² This persistent virus fraction was found to be in the form of an infectious virus - antibody complex (sensitized virus) that could be neutralized by anti-IgG serum, an antibody not directed toward and inactive against the virus particle.³ It is the latter reaction that significantly enhances the sensitivity of the serum neutralization test. For detecting small quantities of antiviral antibody attached to virus and for neutralizing sensitized virus, anti-IgG antibody is highly effective.¹⁰ To explain the phenomenon, it has been postulated that anti-IgG antibodies may form bridges that cover critical infective sites on the virus particle or act by stabilizing the attachment of antiviral antibody to virus.^{11,12} The mechanism, however, by which neutralization of the infectious virus - antibody complex by anti-IgG serum occurs has not been completely resolved.

The high precision and reproducibility of 50% serum-neutralizing end points obtained with the use of the serum neutralization anti-IgG test are a reflection of the assay procedure employed to measure unneutralized VEE virus.¹ Twofold differences in serum-neutralizing titers could be readily distinguished. The utilization of a protocol in which a constant virus quantity is mixed with serial dilutions of antiviral serum provides a better estimate of serum-neutralizing antibodies than that of procedures based on animal death end points¹³ or neutralization indexes.¹⁴ In comparative estimates of VEE neutralizing antibodies in human and monkey sera, the sensitivity of the serum neutralization anti-IgG test was from 300- to 400-fold greater than that of the mouse serum neutralization test. In addition, the ability of the former test to determine serum-neutralizing titers within 24 hours is an important advantage for diagnostic purposes over conventional serum neutralization tests. The principle of the serum neutralization anti-IgG test in conjunction with the immunofluorescent cell counting assay of viruses may be widely applicable to the development and usefulness of similar tests with comparable advantages for detecting and quantitatively measuring serum-neutralizing antibodies of other viral infections.

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